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Scientific Operating Procedure SOP-C

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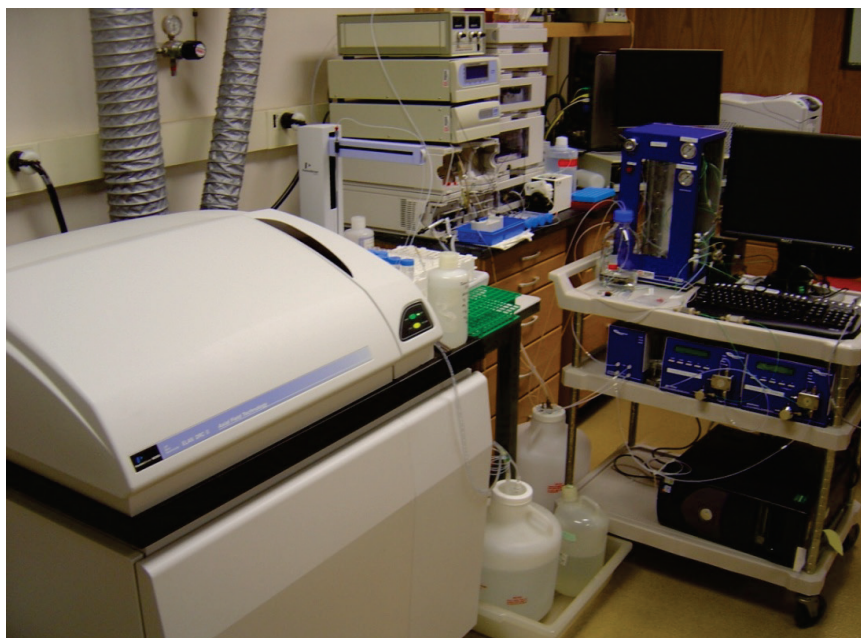
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Final report

Abstract

Characterization of nanomaterials must include analysis of both size and chemical composition. Field Flow Fractionation (FFF) is a powerful tool for determining the size of nanoparticles. Through the use of a combination of common detectors, such as UV-VIS (Ultraviolet-Visible Spectrophotometry) absorbance, with advanced methods, such as ICP-MS (Inductively coupled plasma mass spectrometry), high-resolution nanoparticle sizing and compositional analysis at the µg/L concentration level can be obtained. Single particle counter ICP-MS (SP-ICP-MS) has increased sensitivity compared to Field Flow Fractionation Inductively coupled plasma mass spectrometry (FFF-ICP-MS), with detection and sizing concentrations of ng/L. Such low-level detection and characterization capability is critical to nanomaterial investigations at biologically and environmentally relevant concentrations. The techniques have been modified and applied to characterization of all four elemental constituents of Cadmium Selenide/Zinc Sulfide core-shell quantum dots, silver nanoparticles with gold seed cores, and gold nanoparticles. Additionally, sulfide coatings on silver nanoparticles can be detected as a potential means to determine environmental aging of nanoparticles. Extraction of nanoparticles from tissues is possible using tetramethylammonium hydroxide (TMAH). Though any analysis described above is possible, only SP-ICP-MS has been employed to detect tissue extracts. This special report describes the SOP (Scientific Operating Procedure) for analysis of engineered nanoparticles (ENPs), through the various separation and detection techniques described above. These analytical tools were tested on a variety of gold and silver standard nanoparticles that have been extensively characterized.

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Preface

Acronyms

AF4	Asymmetrical flow Field Flow Fractionation
DLS	Dynamic Light Scattering
DRC	Dynamic Reaction Cell
ENP	Engineered Nanoparticle
FFF	Field Flow Fractionation
ICP-AES	Inductively Coupled Plasma Atomic Emission Spectroscopy
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
kDa	kiloDalton
kg	kilogram
L	Liter
Mg	milligram
mL	milliliter
ms	millisecond
min	minute
ng	nanogram
nL	nanoliter
nm	nanometer
SED	Sedimentation
SEM	Scanning Electron Microscopy
SF4	Symmetrical flow Field Flow Fractionation
SP-ICP-MS	Single Particle Counter ICP-MS
SRM	Standard Reference Material
TEM	Transmission Electron Microscopy
TMAH	Tetramethylammonium Hydroxide
µg	microgram
µL	microliter
µm	micrometer

UV-VIS	Ultraviolet Visible Spectrophotometry
VWD	Variable Wavelength Detector
W	watt

1   Introduction

This scientific operating procedure (SOP) describes how to determine size and composition of nanoparticles, either in stock solutions or after they have been exposed to the environment. When characterization of nanoparticles in solution is desired, nanoparticle concentration is the primary limiting factor; concentrations should be high enough to be detected (generally ng/L to μg/L, depending on the method used as described below), but without carryover issues with instrument contamination (generally observed with mg/L concentrations and above). Nanoparticle characterization in solid media (e.g., soil, sediment, or tissue) first requires extraction of the solid media to an aqueous phase prior to analysis, with subsequent attention to analyte concentration.

Nanomaterials have three relevant metrics: particle size, total number of particles, and mass concentration. Each of these metrics may be of importance for assessing the overall risks associated with these materials. Determinations of each of these metrics requires a suite of analytical techniques, each yielding both the complimentary and sometimes overlapping characterization data necessary to completely describe nanomaterials in a variety of sample types. The present SOP combines best laboratory practices available from the literature and professional experience of ERDC research scientists and collaborators.

2   Background

Complete characterization is critical to understanding the fate, effects, and transport of nanomaterials in the environment. Without a solid under-standing of the physiochemical state of nanomaterials, nothing definitive can be said as to how they change or are changed by environmental exposure. The ability to characterize nanoparticles at environmentally relevant concentrations and in environmentally relevant media is critical to complete nanoparticle characterization. Nanoparticle behavior and effects may be drastically different at ng/L, μg/L, and mg/L concentrations, minimizing the relevancy of extrapolation if attributes are measured at levels which are not observed in the environment being studied.

The most commonly used detection and characterization methods available to assess particle concentration and size distributions include microscopy (Leppard et al. 2005), chromatography (Song et al. 2003), centrifugation (Lyon et al. 2006), laser light scattering (Powers et al. 2007), and filtration (Howell et al. 2006; Akthakul et al. 2005). However, each of these techniques is only capable of directly determining a single metric related to nanomaterials, either particle size or particle mass. The use of these techniques for analysis of engineered nanoparticles (ENPs) in environmental matrices is challenging for two main reasons. The first is that distinguishing ENPs from other constituents of the matrix — such as natural particles, humic substances, and other debris — can pose difficulties (Lead and Wilkinson 2006). The second problem identified with these techniques relates to method sensitivity, which is generally insufficient compared to environmentally and toxicologically relevant concentrations (μg/L to ng/L ranges) (Hassellöv et al. 2008; Kennedy et al. 2010). Despite these drawbacks, these techniques can be used individually or in conjunction with specialized separation techniques (discussed below) to provide information about ENPs in environmental samples, if sample concentration is sufficient to be detected.

One of the most commonly used approaches to visually study nanoparticles is microscopy, either Transmission Electron Microscopy (TEM) or scanning probe microscopy (SEM). Theoretically, microscopy offers the ultimate sensitivity, with the ability to detect/image a single nanoparticle; however, accomplishing this practically is equivalent to the

proverbial "finding a needle in a haystack." Although these techniques provide a visual image of the nature of these particles, some shortcomings of the techniques include the possibility of non-representative sampling, changes during the preparative process (i.e., agglomeration), and inability to find individual particles in very dilute samples.

Another common approach that has long been used to study colloidal solutions is Dynamic Light Scattering (DLS), which measures the particle hydrodynamic diameter. However, limitations for the study of nanoparticles are numerous, including: poor sensitivity at dilute concentrations, nonselective material detection, inability to differentiate between nanoparticles and other matrix components, and difficulty with reliably quantifying the relative proportions of particle or aggregate sizes in multi-modal distributions. Multi-modal populations are particularly problematic for DLS as intensity-normalized results will characteristically be disproportionately skewed to the larger particles/aggregates in suspension even if smaller sizes predominate.

Spectrometry techniques provide an elemental specific detection tool capable of identifying and quantifying elements at environmentally relevant concentrations (ng/L). Although both ICP-MS and ICP-AES can be used to identify metals in an aqueous solution, ICP-MS is more sensitive than inductively coupled plasma atomic absorption spectrometry (ICP-AES). Both techniques are used to analyze aqueous samples and can be run in batch mode with many samples or online with other detectors and sample separation techniques. Due to the formation of polyatomic ion interferences in the plasma ionization step, some elements — such as sulfur or iron — are difficult to analyze by ICP-MS. In this case, a reaction or collision cell type instrument may be required for detection of some elements, or an ICP-AES may prove to be more sensitive in avoiding polyatomic interferences. Both ICP-MS and ICP-AES — run in standard mode — are only capable of determining particle mass distributions.

Recent method development has produced a specific ICP-MS analytical mode, SP-ICP-MS, which allows for the determination of particle size, particle number, and mass concentration of nanomaterials in solutions (Mitrano et al. 2012; Pace et al. 2011). This is the only analytical technique that is capable of determining all three relevant nanomaterial relevant metrics simultaneously. Initial development of SP-ICP-MS was performed by Deguelder et al. (Degueldre et al. 2006), and further method

refinement has been conducted recently (Mitrano et al. 2012; Pace et al. 2011; Laborda et al. 2011; Pace et al. 2012). As SP-ICP-MS is based on introducing individual ENPs into the ICP-MS plasma, this technique requires dilute solutions in order to avoid particle coincidence (the analysis of two particles simultaneously). It is most applicable in the low to mid ng/L range, and is thus ideal for analyzing ENP samples [(Pace et al. 2011; von der Krammer et al. 2012). The main limitation of SP-ICP-MS is the lower size limit of particle detection, which is generally between 20-30 nm for Au and Ag NPs and depends on the ICP-MS instrument used (Laborda et al. 2011; Pace et al. 2012).

Field-Flow Fractionation (FFF) consists of a suite of high resolution sizing techniques, which allows separation and sizing of macromolecules, submicron colloids, and nanoparticles of 1 – 100 nm, depending on the type of field applied and mode of operation (Ranville et al. 1999). The separation process is similar to chromatography, except that it is based on physical forces (e.g., diffusion) as opposed to chemical interactions. Particle separation is performed in a thin channel with laminar flow under the influence of a perpendicular field. There are three common FFF techniques that are the most commercially available, including thermal, sedimentation, and flow FFF. Depending on the type of analysis that is being performed, the FFF technique would be chosen to achieve optimal separation results. Applications of FFF have become increasingly diverse in the recent years, to include separation and characterization of proteins (Liu et al. 2006), polymers (Messaud et al. 2009), cells (Ratanathanawongs-Williams and Lee 2006), natural nanoparticles (Chianéa et al. 2000), and — more recently — manufactured nanoparticles Lead and Smith 2009). Separation techniques, including FFF, impart a dilution factor which can make environmental sample analysis challenging Gray et al. 2012). While sensitive detectors, such as ICP-MS or ICP-AES, have been employed to counteract dilution, detection limits for these approaches are still above predicted environ-mental concentrations for many systems (ng/L) (Gray et al. 2012; Bednar et al. 2013; Poda et al. 2011). At present, the only technique capable of determining particle size, number concentration, and mass concentration for ENP-containing samples in the 1-10 μg/L concentration range (approximate detection limit for FFF-ICP-MS) is single particle ICPMS (SP-ICP-MS) (Gray et al. 2012; Bednar et al. 2013).

3   Scope

The current SOP provides two key pieces of information for nanoparticle characterization: 1) analytical techniques for detecting and quantifying nanoparticles, specifically at low (environmentally relevant, ng/L to µg/L) concentrations, and 2) extraction procedures for solid matrices not amenable to direct analysis by the aforementioned techniques. The methods provided have been tested on a suite of standard nanoparticles, and the base methodologies demonstrated to be effective. Unless otherwise specified, nanoparticle suspensions were created following ERDC/EL SR-15-1 or purchased from a vendor such as NIST. Specifically, test data is provided for NIST Standard Reference Material (SRM) 8012 citrate-stabilized 30 nm gold (Au), Nanocomposix 30 nm silver, and NIST SRM 1898 Titanium Dioxide nanomaterial (TiO$_2$), among others. However, with different nanomaterials, various complex natural matrices, and nanomaterial coatings, method refinements may be needed. Due to the sensitivity of the ICP-MS detector, metal and metal oxide/salt nanoparticles will be the most sensitive, although use of non-selective detectors, such as UV-Vis absorbance, expands the method use (albeit with higher detection limits) to other non-metal nanoparticles while still providing information about metallic particles. Figure 1 gives an overview of the procedures outlined in the current SOP document with references to relevant sections.

Figure 1. Outline of procedures described in the current SOP for characterization of nanoparticles in various matrices.
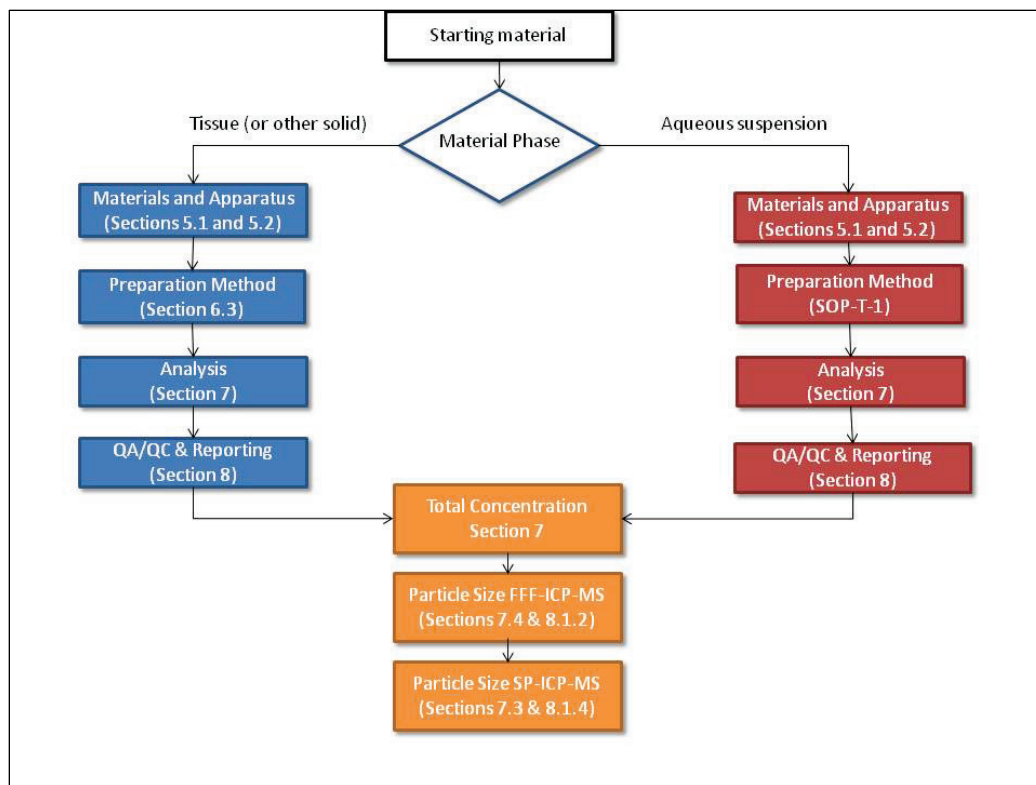
4   Terminology

4.1   Related Documents

Guidance for instrument optimization (e.g., ICP-MS tuning) is provided by the various instrument manufacturers, and standard USEPA methods published for determination of total metals in environmental samples (e.g., SW-846 methods 6010 and 6020). Published methods for FFF and SP analysis may also be consulted that provide additional information for other environmental matrices, nanoparticles, and other analytes of interest (Mitrano et al. 2012; Pace et al. 2011; Pace et al. 2012; Gray et al. 2012; Bednar et al. 2013; Poda et al. 2011; Mitrano et al. 2012; Gray et al. 2013).

4.2   Definitions

- Agglomerate, n — In nanotechnology, an assembly of particles held together by relatively weak forces (for example, Van der Waals or capillary), that may break apart into smaller particles upon processing, for example.
- Coincidence, n – The process of analyzing two particles during one dwell time when running SP-ICP-MS. The resulting particle is observed to have twice the mass and thus overpredicts ENP size (based on observed mass) of what is assumed to be one particle. This effect also affects particle number concentration calculations by underpredicting the number of particles in a solution.

5　Materials and Apparatus

5.1　Materials

- Samples containing ENPs (e.g., Commercial stock solutions, diluted exposure media, biota exposed to NPs, etc.)
- Nanomaterial Standards

  o NanoXact, Nanocomposix (San Diego), various sizes
  o NIST SRMs 8011, 8012, 8013 (50 mg/L Au, various sizes)
  o NIST SRM 1898 TiO_2

- Polystyrene standard particles (20, 50, 100 nm - various suppliers, e.g., PostNova)
- Ultrapure, Deionized Water (NanoPure, MilliQ or comparable product)
- Pipettes of volumetric flasks to perform sample and standard dilutions
- TMAH – 20% w/w aqueous
- Dissolved element ICP-MS/AES standards for any metal of interest
- Test tubes for sample preparation and tissue extractions, (polypropylene)
- Analytical balance for weighing non-aqueous samples
- Optima grade nitric (HNO_3) and hydrochloric (HCL) acids for dissolved standard preparation
- Sonication bath (Fisher Ultrasonic Bath (FS140D, 135W) or equivalent bath)

5.2　Apparatus

5.2.1　Field Flow Fractionation

Separation of ENPs in mixtures should be accomplished using one of the various FFF techniques. Techniques that have been applied to nano-materials include sedimentation FFF (SED), symmetrical flow FFF (SF4) and asymmetrical flow FFF (AF4) (see, for example, references Gray et al. 2012; Poda et al. 2011; Mitrano et al. 2012). SF4 and AF4 FFF are quite similar, with differences existing only in the channel shape and the way flows are applied across the channel. As observed with many analytical techniques, actual analysis conditions will vary between ENP type, sample matrix, and carrier solution. Optimizations must be performed for any FFF technique to ensure adequate particle recovery and ideal interactions between sample ENPs and the FFF membrane exist. Some commonly reported FFF parameters are listed in Table 1.

Table 1. Separation and detection instrument parameters used to measure nanoparticles by FFF techniques. Conditions were specific to the exact nanoparticles used and may vary based on different sample particles.

Symmetrical FFF-UV-ICP-MS/AES System	Postnova F-1000
Membrane	10 kDa regenerated cellulose
Channel and Cross Flow	1.0 and 0.75 mL/min, respectively
Injection Volume	50 μL
Channel Thickness	254 μm
Load Time	15 s
Relaxation Time	3.2 min
Approximate Fractogram Time (100 nm particle elution)	25 min
Asymmetrical FFF-UV-DLS System	Postnova AF-2000 MT
Membrane	10 kDa regenerated cellulose
Channel and Cross Flow	1.0 and 0.75 mL/min respectively
Injection Volume	100μL
Channel Thickness	350 μm
Injection – Focusing Time	8 min
Approximate Fractogram Time (60 nm particle elution)	22 min
UV-VIS Absorbance Detector	Agilent 1100 VWD
Wavelength Monitored	254, 395, 420, or 520 nm
Integration Time	0.4 sec
ICP-MS	Perkin Elmer Elan DRC II
Plasma Power	1250 W
Nebulizer, Spray Chamber, and Flow	MiraMist, Double Pass Scott, 0.85 L/min
Masses Monitored (standard mode)	^{107}Ag, ^{109}Ag, ^{197}Au, ^{111}Cd, ^{66}Zn, ^{82}Se
Masses Monitored (DRC mode)	^{111}Cd, ^{66}Zn, ^{78}Se, ^{48}SO
DRC Reaction Gas and Flow Rate	Ultrapure Oxygen, 0.7 mL/min
RPq for DRC mode	^{111}Cd = 0.75, ^{66}Zn = 0.75, ^{78}Se = 0.75, ^{48}SO = 0.55
Dwell Time per AMU	200 – 500 ms
Readings per Replicate	1200 – 1800
ICP-AES	Perkin Elmer Optima 5300DV
Plasma Power	1400 W
Nebulizer, Spray Chamber, and Flow	MiraMist, Cyclonic, 0.65 L/min
Plasma Viewing Mode	Axial
Wavelengths Monitored	Ag 328.068 nm, Au 267.595 nm , Cd 228.802 nm, Zn 206.200 nm, Se 196.026 nm, S 180.669 nm
Integration Time	5 s
Readings	300
SP-ICP- MS	Perkin Elmer NexION 300 Q
Plasma Power	1600 W
Nebulizer, Spray Chamber, and Flow	Meinhard, Cyclonic, 1 mL/min
Efficiency Calibration	Particle Size Method
Masses Monitored	^{107}Ag, ^{197}Au
Dwell Time per AMU	10 to 0.1 ms
Readings Per Sample	20000

5.2.2 Detection techniques

5.2.2.1 ICP-MS/AES

ICP-MS and ICP-AES are powerful analytical tools that allow for accurate determination of sample metal content at concentrations in the low µg/L or even ng/L range. These instruments are able to detect most elements in a standard analysis mode. The use of a reaction cell or collision cell ICP-MS can improve the signal observed for certain particles (quantum dots, etc.), while ICP-AES can also be used to detect elements with polyatomic interferences. Specific instrument calibration should be performed following manufacturer's instructions prior to running samples. Common operating conditions for ICP-MS or AES instruments are listed below in Table 1.

5.2.2.2 SP-ICP-MS

Analysis of samples using SP-ICP-MS can only be performed using ICP-MS instruments capable of collecting data at or below 10 ms. Dwell times commonly used include 10 and 3 ms with advances in technology allowing the use of 0.1 ms. Also, instruments must be capable of acquiring 200,000 or more readings at a specified dwell time. The number of readings required per sample varies based on sample concentration but should be sufficient to build a particle size distribution. Commonly reported SP-ICP-MS parameters are listed in Table 1.

5.2.2.3 UV-VIS

UV-VIS absorbance detection can be coupled to FFF as an easy-to-use detector, allowing for retention times to be recorded. Observed retention times are related to size based on FFF theory. The selectivity of constituents, which can be detected by UV-V, is limited by the number of wavelengths that each specific detector can monitor during a single run. UV-VIS is most useful as a detector for FFF when using polystyrene beads for retention time size calibration (Bednar et al. 2013; Poda et al. 2011).

6   Procedure

6.1   Sample Separations

The theory behind FFF separation and sizing is well-developed (Giddings 1968; Giddings and Caldwell 1989; Schimpf et al. 2000). One of the advantages of flow FFF for particle size determination is that elution time under identical processing conditions (cross-flow and channel flow settings, carrier solution, etc.) is solely related to particle size (specifically, to hydrodynamic diameter), and follows a linear correlation (Lee et al. 1996). Thus, by changing the FFF flow conditions, different particles sizes, if present in a mixture, can be separated. The retention time of particles can ideally be related to particle diameter, either by using FFF theory or particle size standards. This step should be employed for polydispersed samples or for samples that might contain particles in the size range below 20 nm.

6.2   Aqueous Sample Preparation

Aqueous samples can either be analyzed directly using FFF techniques, or may be diluted in deionized water prior to running a more sensitive technique such as SP-ICP-MS. It is common practice to sonicate samples in a water bath (5 min) to ensure a stable suspension prior to analysis, though this step is not necessary if a stable suspension already exists. For more specifics on preparation of a stable aqueous suspension, please refer to SOP-T-1.

Samples must be diluted in deionized water prior to running SP-ICP-MS as this technique requires dilute (10 μg/L to 10 ng/L) particle mass concentrations depending on particle size. Some consideration must also be given to the specific matrix that a sample is in. Analysis with DLS, UV-Vis or ICP-MS/AES could all be affected by matrix effects. Adequate blanks must be included to identify these effects if they occur, followed by matrix matching or other background reduction approaches.

6.3   Tissue Sample Preparation

If stable aqueous samples are already obtained, skip to step 7.0. If dispersion of separated nanoparticles is required, refer to SOP-T-1.

Tissue samples must be digested prior to analysis in order to release all ENPs in a tissue sample into the aqueous phase. To perform the tissue extraction, samples are digested in a solution containing 20% tetramethylammonium hydroxide (TMAH), which has a pH of approximately 13.5. This organic base completely digests tissues while leaving particles intact. For all tissue extractions, a solvent to tissue ratio of 20:1 should be maintained to ensure reproducibility using different tissue masses and types.

The 20% TMAH solution should be added to a TMAH-compatible test tube or an otherwise acceptable extraction container containing a tissue sample of known mass. This mixture of TMAH and substrate should be sonicated (at approximately 135 W) in a water bath for 60 min at room temperature prior to sitting for 23 hours at room temperature. For ENPs that are light sensitive, samples should be maintained in the dark for the remainder of the extraction. Following the 24-hour extraction period, samples should be diluted to a maximum TMAH concentration of 2% prior to analysis. Any remaining sample processing should follow the aqueous sample preparation step, 6.2.

6.4   Sample Analysis

A variety of techniques can be used to analyze aqueous samples containing ENPs, each providing different, yet complementary, information about them. For example, DLS and FFF provide hydrodynamic diameter while SP-ICP-MS and TEM provide information about the primary particle size. Detection techniques include DLS, UV-Vis, ICP-MS, ICP-AES and SP-ICP-MS, and FFF (coupled to a variety of detectors, such as ICP-MS, ICP-AES, and UV-VIS absorbance. This SOP is dedicated to FFF and SP-ICP-MS analyses, although they are complimentary to other techniques listed above.

The SP-ICP-MS analysis technique utilizes the sensitivity of ICP-MS to detect individual particles; therefore directly measuring the particle number concentration of a sample. Further, through calibration, the mass of an observed particle can be converted to a size distribution using an assumption about particle geometry (usually determined by TEM).

7   Analysis

7.1   ICP-MS

Aqueous samples are aspirated into the ICP-MS using a peristaltic pump at a flow rate that can range from 0.2-1.0 mL/min, depending on pump tubing size and instrument conditions. Parameters outlined in USEPA method SW-846 6020 are useful in optimizing the instrument prior to method refinement for the specific analyses to be performed. In standard mode, the number of ions counted per second is related to a standard calibration curve constructed for dissolved ions. Observed signal intensity in a sample is then related to a concentration using the dissolved element calibration curve.

7.2   ICP-AES

Aqueous samples are aspirated into the ICP-MS using a peristaltic pump at a flow of about 1.0 mL/min. Parameters outlined in USEPA method SW-846 6010 are useful in optimizing the instrument prior to method refinement for the specific analyses to be performed. The number of photons counted per second is related to a standard dissolved ion curve. Observed signal intensity in a sample is then related to a concentration using the dissolved element calibration curve.

7.3   SP-ICP-MS

SP-ICP-MS analysis procedure should follow the procedure described by Pace et al. (2011). All analyses are performed using aqueous samples and standards. Dissolved standards bracketing the number of counts produced by an individual particle should be made for every element of interest. A standard particle, usually NIST SRM8013 (60 nm Au), is used to calculate the transport efficiency daily due to the lower size limit of this technique being approximately 30 nm. This transport efficiency, determined using either the particle number or particle size approach (Pace et al. 2011), is then applied to all subsequent samples, regardless of their elemental composition. Instrument dwell times commonly used for SP-ICP-MS include 10, 3, and 0.1 ms; however, dwell times within this range are acceptable. Short dwell times allow entire particles to be analyzed, but prevent more than one particle from being analyzed at the same time (coincidence). To be considered a particle, the intensity of an observed

pulse must be greater than 3σ of the background signal. Each particle (observed as counts) is then converted to a diameter following the method proposed by Pace et al (2011). The observed number of pulses, corrected for efficiency, is the particle number concentration while the integrated observed counts for each sample yields the particle mass concentration.

7.4   FFF-UV-VIS or FFF-ICP-MS/AES

This SOP utilizes exclusively external calibration of the FFF retention time using polystyrene beads that are NIST-traceable. A mixture of the three bead sizes (20, 50, and 100 nm) are injected into the FFF using the 6-way valve. Under conditions used to obtain the data described below (cross flow of 0.5-0.75 mL/min and channel flow of 1.0mL/min), the FFF software will use a relaxation time of approximately 3.5-5 minutes. During this time, injected particles are pushed against the membrane before channel flow is restarted to begin the hydrodynamic separation process. Data for the polystyrene beads are collected with a UV-VIS absorbance detector at 254 nm, and an EXCEL plot of retention time vs particle size is created (Bednar et al. 2013; Poda et al. 2011). The FFF technique can then be coupled to either an ICP-MS or ICP-AES for more sensitive detection of the ENPs (Bednar et al. 2013; Poda et al. 2011). The particle size calibration curve generated from the FFF-UV-Vis retention time analysis is subsequently used to determine unknown ENP particle sizes by comparison of retention times measured by FFF-ICP-MS/AES (for metal nanoparticles).

8   Reporting

8.1. FFF-UV-VIS

UV-Vis can be used to detect ENPs coupled to FFF if concentrations are sufficiently high (usually mg/L). UV detectors are quite variable as some are able to analyze single wavelengths at a given time, while others having the ability to scan multiple wavelengths at the same time. The applicability of this technique is highlighted in Figure 2 and Figure 3. An example of this technique shows three overlays of polystyrene particles in addition to a void peak (material not retained by the field) all injected over a 24-hour period (Figure 2). Retention time was plotted against diameter, resulting in a correlation coefficient greater than 0.9999, indicating that FFF- UV-Vis is excellent at predicting size reproducibly for polystyrene particles. The use of this separation and analysis technique is further proven using standard Au and Ag ENP (Figure 3). The size-dependent absorbance of Au and Ag nanoparticles, assumed related to surface plasmon resonance (Heath 1989; Xiong et al. 2005), makes it difficult to relate the UV-VIS peak area obtained in FFF-UV-VIS analysis to the mass of particles eluting across the fractogram. One desirable feature of FFF is the ability to quantify the amounts of NPs in mixtures, thus one can see that it would be very difficult, if not impossible, to relate AF4-UV-VIS response to mass for complex NP mixtures. However, determination of size distributions is possible.

8.2   FFF-ICP-MS/AES

Use of ICP-MS and ICP-AES detectors' elemental specificity and sensitivity allows simultaneous detection of six nanoparticle types (3 Au and 3 Ag), mixed in a single solution as shown in Figure 3 below. Figure 3 shows SF4-UV-VIS, SF4-ICP-MS, and SF4-ICP-AES fractograms of nominally 10, 30, and 60 nm silver and gold particles. In contrast to the UV-VIS absorbance data (Figure 3), however, use of ICP-MS and ICP-AES detectors' elemental specificity allows simultaneous detection of all six nanoparticle types mixed in a single solution. When comparing only ICP-MS and ICP-AES results, the superior detection capabilities of the ICP-MS are clearly evident in Figure 3. The peak height for the ICP-MS fractogram suggests that a 1:20 dilution would still produce discernable peaks, resulting in estimated detection limits near 10 µg/L as previously reported (Bednar et al. 2013; Poda et al.

2011; Pace et al. 2010). The use of NIST gold particles for size calibration (with or without ICP-MS or ICP-AES detection) in place of polystyrene beads and UV-VIS absorbance detection is possible based on these results; however, agreement between both types of size standards and detectors provides validity to the methodology.

Figure 2. Overlay of triplicate FFF-UV-VIS fractograms of polystyrene bead calibration standards. FFF separation conditions were 1.0 mL/min channel flow and 0.75 mL/min cross flow. UV-VIS absorbance detection is at 254 nm wavelength. [Inset]: Linear regression calibration function using 20, 50, and 100 nm polystyrene bead standards. Error bars represent standard deviation of the triplicate retention times obtained from UV-VIS absorbance data at maximum absorbance.
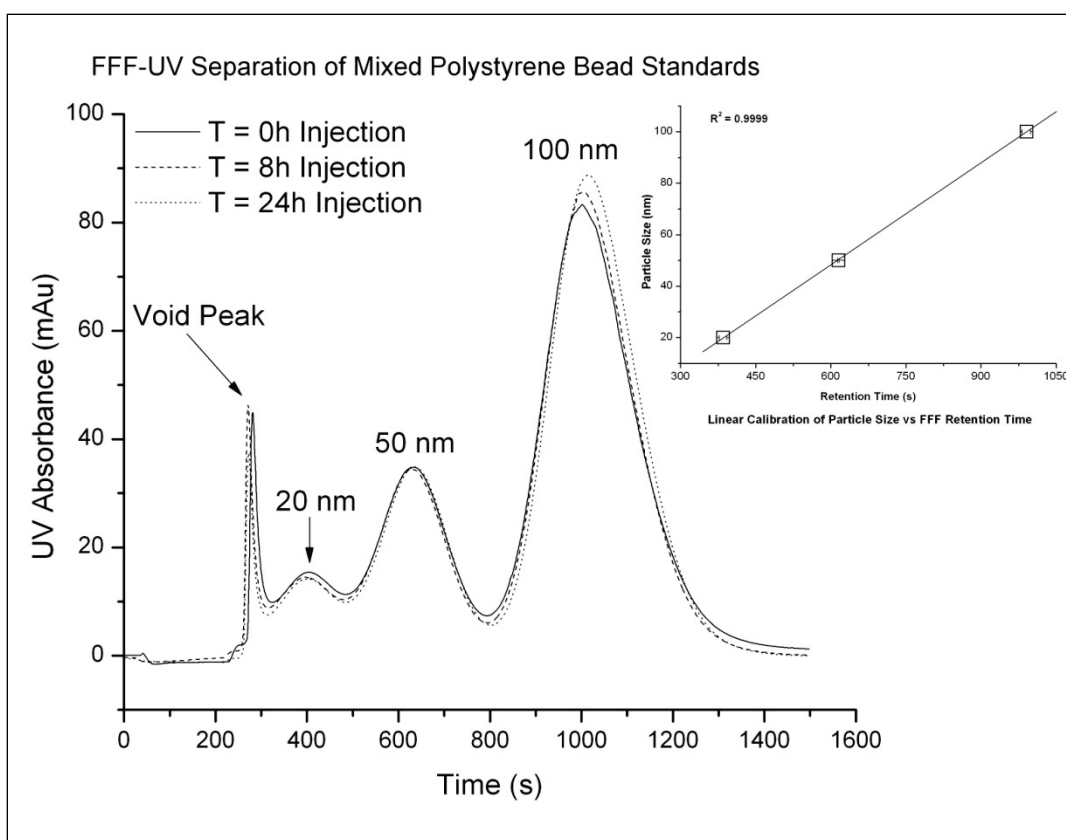
Figure 3. FFF-UV-VIS (A), FFF-ICP-MS (B), and FFF-ICP-AES (C) fractograms of 6 nanoparticle types at 200 µg/L concentration for each particle. The FFF-UV-VIS fractograms are composites plotted from 4 individual analyses of 3 particle mixtures (Nanogold and Nanosilver at 2 wavelengths), whereas the fractograms using the ICP-MS and ICP-AES are single analyses of a mixture of all 6 nanoparticles. The FFF-ICP-MS determined sizes for gold and silver were 18 and 18, 34 and 37, and 62 and 69 nm, respectively. The 30 nm gold and silver samples are NIST SRM 8012 30 nm gold, Nanocomposix 30 nm silver.
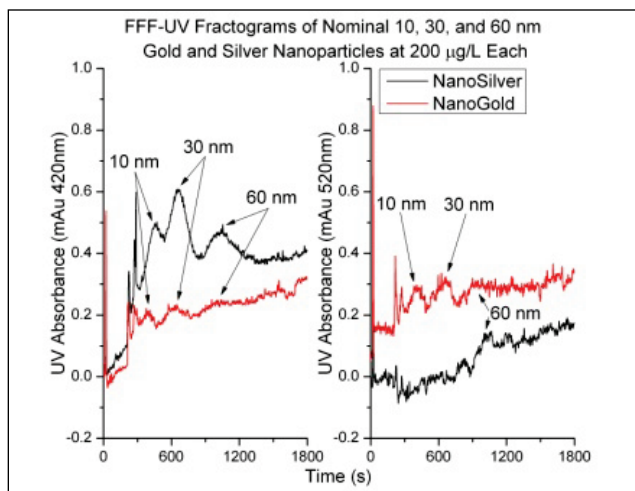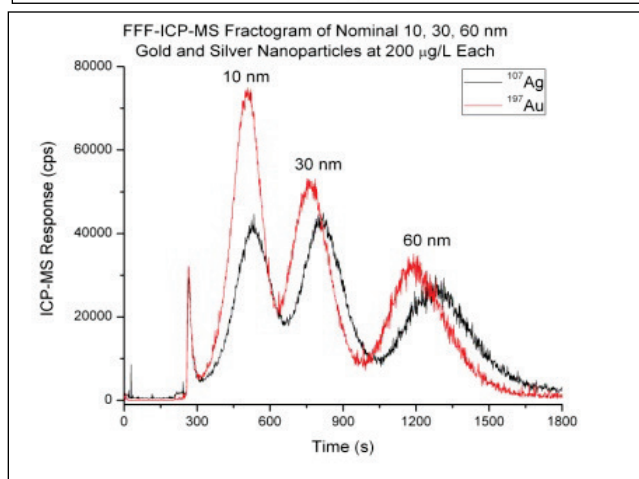
Mixtures containing 10, 30, and 60 nm particles represent an ideal mixture with plenty of separation between monodisperse particles. ENPs in environmental samples will likely have much broader size distributions and thus FFF-ICP-MS was tested over a greater range of particles. Overlays of FFF fractograms using individual NanoXact particles obtained under the standardized FFF flow conditions are shown in Figure 4 (Table 1, SF4-ICP-MS). Though there is not 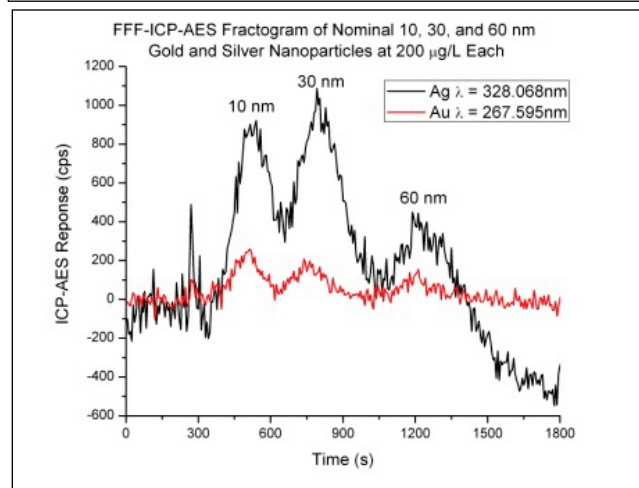clear resolution between particles only separated by 10 nm (TEM measured size), maximum peak response indicates that peak maxima can be used to differentiate between different particle sizes. The only exception observed was an overlap between 60 and 70 nm particles. However, the nominal 60 and 70 nm particles shown in Figure 4 are in agreement with the DLS results with sizes reported (Not Shown). This is clearly demonstrated as the fractograms nearly overlap, indicating the similar size of these two nanoparticles.

Figure 4. Individual FFF-ICP-MS fractograms overlain of NanoXact silver nanoparticles. Each peak represents 200 µg/L total silver as particles. FFF separation conditions were 1.0 mL/min channel flow and 0.75 mL/min cross flow with ICP-MS detection using ^{107}Ag.
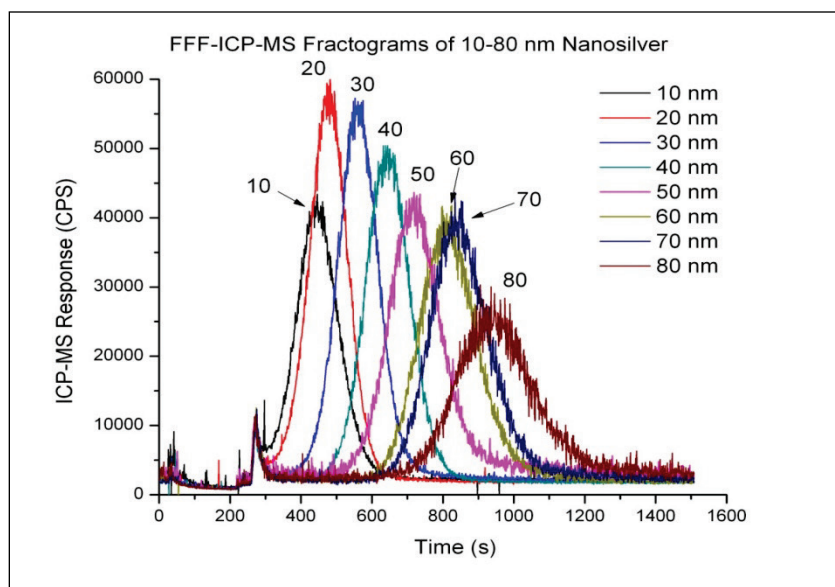
The FFF-ICP-MS method has also been applied to NIST SRM 1898 TiO_2, as shown in Figure 5 below. Two suspensions were prepared according to ERDC/EL SR-15-1, and analyzed within 1 day of preparation. There were no nanoparticles observed in either preparation of the TiO_2 without sonication for an additional five minutes immediately prior to the FFF analysis (Figure 5). The broad fractogram peaks show a large polydisperse population of nanoparticles centered around 90 nm. The suspension

appears to be visually stable (i.e., very little — if any — material settles out), yet there appears to be no detectable nanoparticles without additional sonication immediately prior to analysis.

Figure 5. FFF-ICP-MS fractograms overlain of two preparations of SRM 1898 nanoparticles, with and without additional sonication prior to analysis. FFF separation conditions were 1.0 mL/min channel flow and 0.5 mL/min cross flow with ICP-MS detection using ^{47}Ti.
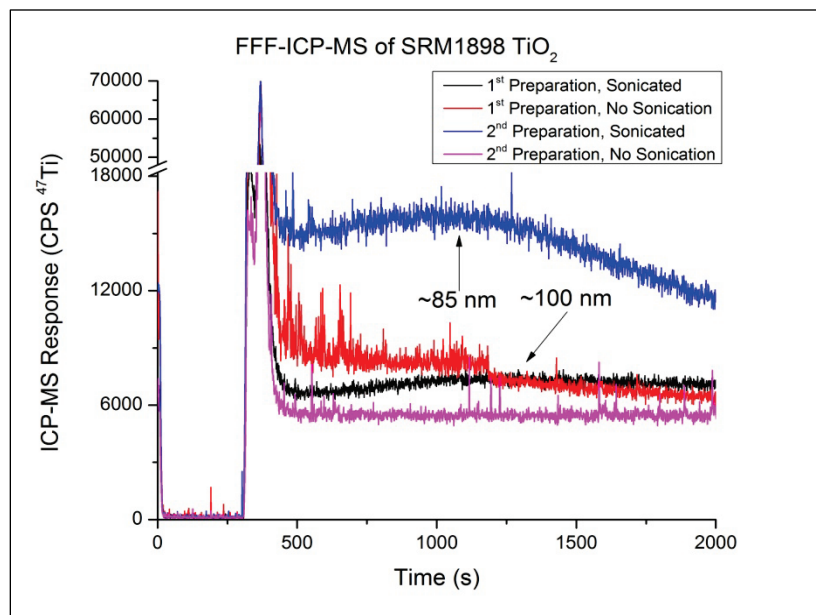
8.3 TMAH Tissue Extraction

Because all three analytical techniques require aqueous solutions for particle size analysis, solid media require extraction prior to nanoparticle determination. Traditional acid extraction techniques for metals are generally sufficient for total metals analysis, yet the aggressive reagents used will often destroy the nanoparticles, creating dissolved ionic species.

The addition of tetramethylammonium hydroxide (TMAH) to an organic substrate (e.g., tissue) should yield a viscous solution in 24 hours, thereby releasing the nanoparticles. The solution should be transparent, though color will depend on the exact tissue that has been digested. This tissue extraction procedure has only been tested using *D. magna*, *L. variegatus* and lean ground beef. Recovery in these matrices varied between 80-120% (Table 2). Further, the use of TMAH, a strong base, did not change the observed size distributions of any test ENP, indicating that this procedure will not alter the primary particle distribution as a strong acid would. Extraction of ENPs from different tissue and solids types should be investigated for particle mass and size recovery prior to analysis.

Table 2. Particle number- and particle mass-based recoveries of 100 nm Au and Ag ENPs (PVP coated) extracted from different biological tissues using TMAH. Beef and L. variegatus were spiked at 98 µg/kg ww Au while Ag was spiked at 19 µg/kg ww. D. magna were spiked at 28 mg/kg dw Au and 5.3 mg/kg Ag.

Tissue Matrix	Particle Number Recovery % ±SD	Particle Mass Recovery % ±SD
Au		
Fresh GB	94 ± 3	89 ±3
Aged GB	89 ±4	88 ±2
D. magna	95 ±2	109 ±4
L. variegatus	95 ±3	95 ±3
Ag		
Fresh GB	95 ±3	104 ±3
Aged GB	107 ±2	121 ±4
D. magna	84 ±4	105 ±8
L. variegatus	95 ±3	107 ±7

8.4   SP-ICP-MS

SP-ICP-MS is capable of determining particle size, particle number, and particle mass concentration for an aqueous sample while simultaneously being able to distinguish a dissolved signal from an ENP signal. The benefit of SP-ICP-MS over FFF-ICP-MS/AES is increased sensitivity by at least two orders of magnitude, allowing characterization of nanoparticles at the parts per trillion range. The current limitations of SP-ICP-MS are for particles below 20 nm as they cannot be detected easily (Pace et al. 2011). For example, TMAH extraction has been used on tissue samples containing multiple ENP sizes as well as samples containing both dissolved and ENP Ag ENPs (Gray et al. 2013). Mixture experiments were conducted using 60 and 100 nm Ag ENPs spiked into tissue samples at identical particle number concentrations. The resulting extracted size distributions compare well in both peak shape and size, demonstrating that SP-ICP-MS can be used to analyze mixtures of particles in both water and tissue extracts (Figure 6). Further, the TMAH does not impact the primary particle size of 60 or 100 nm Ag ENPs as compared to the particles analyzed in water. In a different experiment, dissolved and ENP Ag were compared to assess the ability of SP-ICP-MS to distinguish different forms of the same element. An example of being able to delineate extracted material vs. dissolved material of the same element (Figure 7) is for a pair of Ag samples where one sample is spiked with both dissolved Ag and ENP Ag, while the second sample only shows dissolved Ag. These results show a clear ability of the extraction coupled to SP-ICP-MS to distinguish between multiple forms of a single element, and are evidence that the extraction will not form particulate Ag from dissolved Ag.

Figure 6. Overlay of 60 and 100 nm Ag ENPs (PVP) analyzed in water (A) compared to 60 and 100 nm Ag ENPs extracted simultaneously from ground beef (B).
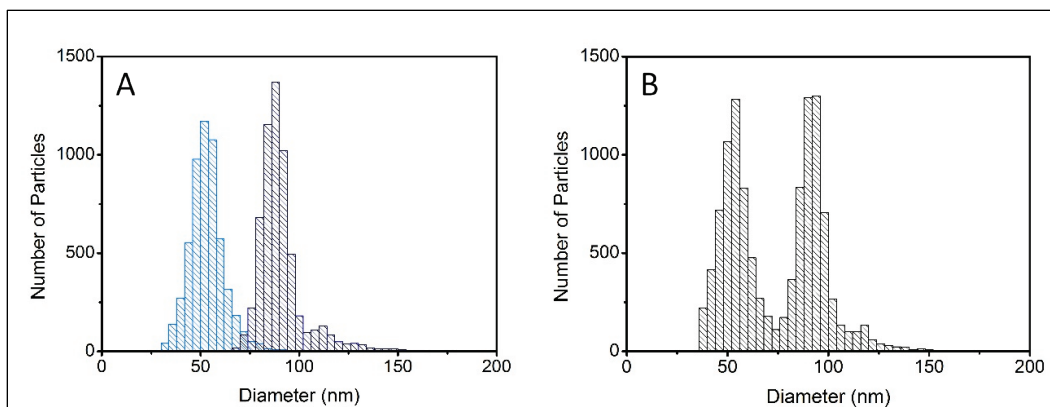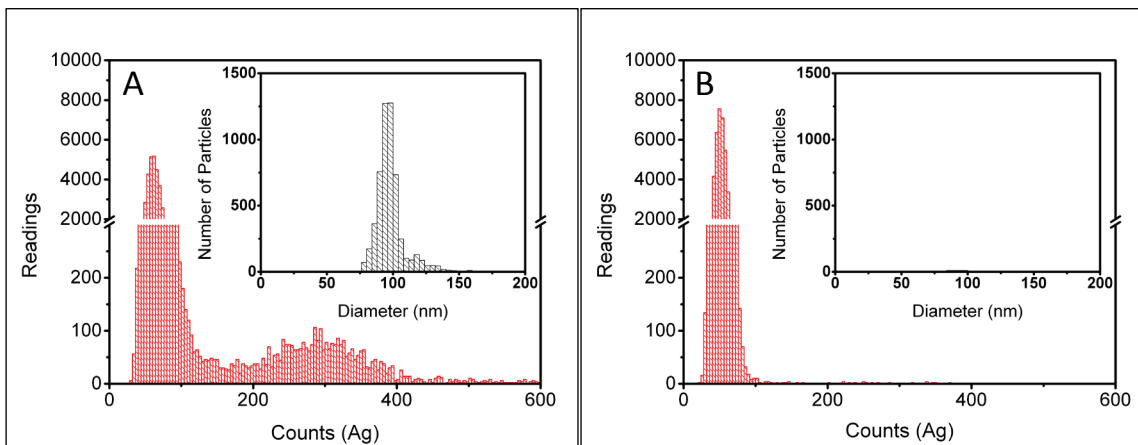

Figure 7. Extraction of dissolved and ENP Ag (100 nm) from beef (A) compared to extraction of dissolved Ag only from beef (B), with converted size distribution in inlay.

9   Key Results Provided

9.1   FFF-ICP-MS/AES

Clear resolution of 10, 30 and 60 nm gold and silver nanoparticles was achieved using ENP Ag and Au ENPs in the same size range (Figure 3). Coupling FFF to ICP-MS/AES can provide concentration information as well as size, if the proper quantitation standards are analyzed and an estimate of analyte loss through and to the membrane can be determined. This is a far more complex issue, and therefore only size distribution is determined using both FFF and the specific spectrometry techniques described here. The SF4-ICP-MS-determined sizes for gold and silver were 18 and 18, 34 and 37, and 62 and 69 nm, respectively. These values agree reasonably well with size determinations in single particle solutions by DLS for gold (31, 36, and 60 nm) and silver (22, 41, and 67 nm) (Bednar et al. 2013; Poda et al. 2011). Stable suspensions for SRM 1898 TiO_2 were limited, and size ranges detected were 85-100 nm — slightly smaller than reported for the material by NIST.

9.2   TMAH Tissue Extraction

TMAH extraction of biological tissues extracted ENPs without significant modification from ground beef, *L. variegatus* and *D. magna*. Recovery of spiked particles was between 80-120%, using both a mass and a number based approach. TMAH did not induce a size change in the ENPs as compared to water, making this tissue extraction technique extremely efficient for recovery of ENPs from tissues.

9.3   SP-ICP-MS

The recently developed analytical technique of SP-ICP-MS is capable of determining particle size, number, and mass concentrations. Gray et al. (2013) used this approach combined with the tissue extraction procedure described in 9.1.2 to detect and quantify multiple ENPs in aqueous samples and tissue extracts. The utility of this extraction and analysis technique has been proven in two ways. First, SP-ICP-MS is sensitive enough to baseline resolve 60 and 100 nm Ag ENPs in tissue extracts (Figure 6). Resolution is based on ICP-MS sensitivity, and will likely decrease as the difference in particle diameter decreases. Second, this technique is capable of resolving dissolved and nanoparticlate forms of a

single element (Figure 7). Using the SP-ICP-MS equations described by Pace et al. (2011), size distributions, particle number distributions, and mass distributions can be obtained and reported. The results of this technique, though powerful, sensitive, and accurate should always be corroborated using at least one other analytical method.

9.4   QA/QC Consideration

Replication of analytical results is crucial to providing adequate assurance of data quality. An excellent demonstration of the reproducibility of the FFF technique is for triplicate FFF-UV-VIS fractograms of polystyrene bead calibration standards (Figure 1). Matrix spike recoveries for SP-ICP-MS track closely with accepted total metals analysis limits of 80-120%. Standard reference materials, generally NIST traceable, are used to calibrate and verify method accuracy.

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14. ABSTRACT

Characterization of nanomaterials must include analysis of both size and chemical composition. Field Flow Fractionation (FFF) is a powerful tool for determining the size of nanoparticles. Through the use of a combination of common detectors, such as UV-VIS (Ultraviolet-Visible Spectrophotometry) absorbance, with advanced methods, such as ICP-MS (Inductively coupled plasma mass spectrometry), high-resolution nanoparticle sizing and compositional analysis at the mg/L concentration level can be obtained. Single particle counter ICP-MS (SP-ICP-MS) has increased sensitivity compared to Field Flow Fractionation Inductively coupled plasma mass spectrometry (FFF-ICP-MS), with detection and sizing concentrations of ng/L. Such low-level detection and characterization capability is critical to nanomaterial investigations at biologically and environmentally relevant concentrations. The techniques have been modified and applied to characterization of all four elemental constituents of Cadmium Selenide/Zinc Sulfide core-shell quantum dots, silver nanoparticles with gold seed cores, and gold nanoparticles. Additionally, sulfide coatings on silver nanoparticles can be detected as a potential means to determine environmental aging of nanoparticles. Extraction of nanoparticles from tissues is possible using tetramethylammonium hydroxide (TMAH). Though any analysis described above is possible, only SP-ICP-MS has been employed to detect tissue extracts. This SOP (Scientific Operating Procedure) describes the analysis of engineered nanoparticles (ENPs), through the various separation and detection techniques described above. These analytical tools were tested on a variety of gold and silver standard nanoparticles that have been extensively characterized.

15. SUBJECT TERMS

Engineered Nanoparticles	Inductively coupled plasma mass spectrometry	Nanoparticle characterization
Field Flow Fractionation	Nanomaterials	Nanotechnologies
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